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Award Number: W81XWH-05-1-0051

TITLE: Anti-Angiogenic Action of Neutral Endopeptidase

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REPORT DATE: November 2006

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/11/06	Annual	1 Nov 2005 – 31 Oct 2006

4. TITLE AND SUBTITLE

Anti-Angiogenic Action of Neutral Endopeptidase

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0051

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Weill Medical College of Cornell University
New York, NY 10021

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: Please see attached.

15. SUBJECT TERMS
Angiogenesis, Cell surface peptidase, Neutral endopeptidase, Basic fibroblast growth factor, Prostate cancer, Proteolysis, Vascular endothelial cells

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
U	U	U	UU	15	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Abstract

Angiogenesis, or the formation of new blood vessels from existing vasculature is an important event in tumor progression. It results from a complex, multistep biochemical cascade that is initiated by the activation of endothelial cells in response to angiogenic factors. In prostate cancers, angiogenic factors are produced by epithelial and stromal cells, and are believed critical to prostate cancer growth and progression. One of the most important of these factors is basic fibroblast growth factor (bFGF), which plays an important role in angiogenesis through the stimulation of endothelial cell proliferation, migration, and protease production *in vitro* phenomenon. A number of studies both *in vitro* and in patient specimens suggest that enhanced expression of bFGF contributes to more aggressive prostate cancer. Clearly, a better understanding of the pathways regulating angiogenesis in the prostate and how these pathways change during malignant transformation and prostate cancer progression will assist in developing more effective therapies for patients with prostate cancer.

Cell-surface peptidases are the guardians of the cell against small stimulatory peptides, functioning to control growth and differentiation in normal cells by regulating peptide access to their cell-surface receptors. They are integral membrane proteins with their enzymatic site exposed to the external cell surface, Neutral endopeptidase (NEP) is a cell-surface peptidase normally expressed by prostatic epithelial cells, whose expression is lost in over half of prostate cancers. NEP substrates include small peptides that have been implicated in prostate cancer progression, including endothelin-1, bombesin and neurotensin. We have now reported that bFGF is also a substrate for NEP.

The goals of this application focus on deciphering the interaction between NEP and bFGF. We have now identified precisely how and where NEP cleaves bFGF, and the biological effects of these cleavage products on prostate cancer cells and human vascular endothelial cells. We are in the process of developing angiogenesis-based models in mice that have been engineered to lack the NEP gene allowing us to directly determine the contribution of NEP to angiogenesis. Studies are ongoing to assess the role of hypoxia on NEP action, and establish the relationship between NEP and other angiogenic factors such as heparin sulfate proteoglycans, vascular endothelial growth factor, and endothelin-1; and in epithelial and endothelial cells

The results generated by our research are likely to contribute to a greater understanding of prostate cancer angiogenesis, and to explain, at least in part, the impact of NEP loss on bFGF expression. Moreover, this understanding potentially will have widespread applicability to not only other cancers, but to other angiogenic processes such as wound healing and ischemic vascular disease, where augmentation of angiogenesis (through inhibition of NEP) would be of therapeutic benefit. This improved understanding of NEP, bFGF and angiogenesis in prostate cancer may then be used in the design of novel therapeutic approaches.

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Introduction

Neutral endopeptidase 24.11 (NEP, CD10, neprilysin) is a 90-110 kD cell surface peptidase normally expressed by a variety of tissues, including epithelial cells of the prostate, kidney, intestine, endometrium, adrenal glands and lung. This enzyme cleaves peptide bonds on the amino side of hydrophobic amino acids and inactivates a variety of physiologically active peptides, including atrial natriuretic factor, substance P, bradykinin, oxytocin, Leu- and Met-enkephalins, neurotensin, bombesin, endothelin-1 (ET-1), and beta amyloid. Loss or a decrease in NEP expression have been reported in a variety of malignancies, including renal cancer, invasive bladder cancer, poorly differentiated stomach cancer, small cell and non-small cell lung cancer, endometrial cancer and prostate cancer (Osman et al., 2004; Papandreou et al., 1998). Reduced expression of cell-surface peptidases such as NEP results in the accumulation of higher peptide concentrations that mediate neoplastic progression (Nanus, 2003).

Using prostate cancer as model to study the involvement of NEP in malignancy, we have demonstrated the following: 1) NEP protein expression is absent in nearly 50% of primary PCs (Osman et al., 2004); 2) NEP inhibits neuropeptide mediated cell growth, cell migration, and ligand-independent activation of the insulin-like growth factor-1 receptor (IGF-1R) leading to Akt phosphorylation (Papandreou et al., 1998; Sumitomo et al., 2001); 3) NEP can inhibit cell migration independent of its catalytic activity via protein-protein interaction of NEP's cytoplasmic domain with tyrosine-phosphorylated Lyn kinase, which then binds the p85 subunit of phosphatidylinositol 3-kinase (PI3-K) resulting in an NEP-Lyn-PI3-K protein complex. This complex competitively blocks the interaction between focal adhesion kinase (FAK) and PI3-K (Sumitomo et al., 2000); 4) NEP directly binds to ezrin/radixin/moesin (ERM) proteins resulting in decreased binding of ERM proteins to the hyaluronan receptor CD44 such that cells expressing NEP demonstrate decreased cell adhesion and cell migration (Iwase et al., 2004); 5) NEP directly interacts with the PTEN tumor suppressor protein, recruiting endogenous PTEN to the cell membrane, leading to prolonged PTEN protein stability and increased PTEN phosphatase activity, and resulting in a constitutive down regulation of Akt activity (Sumitomo et al., 2004); and 6) NEP expression inhibits tumorigenicity in an animal model of PC (Dai et al., 2001). Taken together, these studies have demonstrated that NEP protein functions to suppress and inhibit many processes that contribute to neoplastic progression.

In addition to epithelial cells, enzymatically active NEP is also expressed by vascular endothelial cells of venous and arterial origin (Llorens-Cortes et al., 1992). The NEP substrate ET-1 has previously been shown to act directly on endothelial cells via the ET_B receptor to modulate different stages of neovascularization, including proliferation, migration, invasion, protease production and morphogenesis, resulting in neovascularization *in vivo* (Bagnato and Spinella, 2003). Based on these observations, we investigated whether NEP also functions as an antagonist of angiogenesis. We report that NEP is indeed antiangiogenic *in vivo*, significantly inhibiting angiogenesis. Surprisingly, we demonstrate that NEP catalytically inactivates the potent angiogenic factor basic fibroblast growth factor (bFGF; FGF-2), and that this is antagonized by heparin sulfate, consistent with a modulatory role for heparan sulfate proteoglycans. This is the first report of an enzyme that cleaves bFGF *in vivo*, further demonstrating the potent tumor suppressive actions of NEP. NEP expression in PC cells is negatively regulated by hypoxia, consistent with its antiangiogenic function.

Body

a. Specific aims

The objective of this research is to continue to elucidate the mechanisms by which neutral endopeptidase 24.11 (NEP) functions to inhibit angiogenesis. These proposed studies should provide significant new knowledge on the multifunctional role of NEP as an inhibitor of bFGF-induced angiogenesis as well as clarify the role of the N-terminal cytoplasmic domain with regard to its regulation of angiogenesis. Aim two now includes murine endothelial cells. The specific aims are:

(1) To characterize the interaction between NEP and bFGF.

(2) To delineate the anti-angiogenic action of NEP on vascular endothelial cells of mouse and human origin.

b. Studies and Results

During the second year of grant support, we have continued studies analyzing the antiangiogenic role of NEP on vascular endothelial cells. We have now published the observation that the basic fibroblast growth factor is a substrate for NEP, and demonstrated mechanistically that proteolytic cleavage of bFGF by NEP abrogates signaling by producing inactive cleavage products.

AIM I: Characterization of the interaction between NEP and bFGF.

Identification of a NEP cleavage site on bFGF. To test the hypothesis that NEP could regulate angiogenesis in vivo, we used the murine corneal pocket assay to study the effect of recombinant NEP (rNEP) on bFGF-induced neovascularization. In this assay, pellets containing various concentrations of bFGF were implanted in the cornea ~1 mm from the limbus and neovascularization measured five days later. As shown previously, rNEP significantly inhibited bFGF-induced neovascularization ($P<0.01$). These results suggested the possibility that bFGF was inactivated by NEP via catalytic processing of the bFGF protein. We have also tested VEGF in the corneal pocket model and demonstrated that NEP does not inhibit VEGF-dependent angiogenesis, further defining a specific role of NEP in regulating bFGF-dependent angiogenesis (Figure 1). To assess the physiologic relevance of bFGF cleavage by NEP, we perfomed an *in vitro* kinetic analysis using a 30:1 substrate:enzyme ratio. (Figure 2). The rate of cleavage observed is comparable to that of another known NEP substrate, amyloid-β.

Assess the role of heparanoids in regulating bFGF susceptibility to proteolytic cleavage. Basic FGF is primarily stored in the extracellular matrix and basement membrane associated with heparan sulfate proteoglycan (HSPG). Activity of bFGF is controlled in part by a low-affinity but high-capacity interaction with HSPG. Free bFGF may be proteolytically degraded, as suggested by in vitro reactivity of the C-terminal portion of bFGF to trypsin and chymotrypsin (Kajio et al., 1992; Sommer and Rifkin, 1989). We hypothesized that HSPG binding could function to protect bFGF from degradation by NEP since leucine 135 and glycine 136 of the bFGF protein lie within a basic region where heparin-derived tetra- and hexasaccharides have been reported to complex with bFGF (Faham et al., 1996). Studies are ongoing using sodium chlorate to inhibit heparan sulfate

proteoglycan sulfation, known to reduce bFGF signaling, in conjunction with the pharmacologic inhibitor of NEP CGS24592.

Characterize the actions of bFGF cleavage products on FGF receptor signal transduction pathways, and on the biology of human vascular endothelial cells. Our data reported in last years' progress report has now been published. Currently experiments are being conducted to examine the biological activities of bFGF cleavage products expressed as GST-fusion proteins.

(2) To delineate the anti-angiogenic action of NEP on vascular endothelial cells of mouse and human origin.

Assess the pro-angiogenic activity of bFGF in NEP null mice compared to wild-type mice. Experiments utilizing the murine corneal pocket assay in wildtype mice have been completed and reported (see aim 1 above). As a complementary strategy, we isolated endothelial cells from heart using immunoselection with magnetic beads (Dynal). The cells were able to take up DiI-LDL, form arrays in matrigel (Figure 3). While the wildtype cells exhibit normal cobblestone morphology at confluence and expressed NEP, the NEP-deficient cells appear bizarre morphologically, almost as if they were transformed. To this end, we tested wildtype and knockout endothelial cells for an ability to form colonies on soft agar under standard culture conditions and found that while the wildtype endothelial cells did not grow in soft agar, the knockout cells formed colonies much like LNCaP cells did (Figure 4). Furthermore, NEP-deficient cells had higher levels of basal ERK-phosphorylation (Figure 5). Experiments are underway to characterize responsiveness to bFGF of the two cell lines.

Decipher the hypoxic-dependent of NEP expression in human vascular endothelial cells. Experiments to characterize the negative regulation of NEP activity by hypoxia are ongoing. In preliminary experiments, we have demonstrated using quantitative real time RT-PCR that hypoxia reduces expression of NEP transcripts in LNCaP by approximately 50 % (Figure 6). We hypothesize that this is mediated by Hypoxia inducible factor 1-alpha (HIF-1a) repressive element in the NEP gene and plan to test this hypothesis by examining NEP expression and activity in the presence of transfected HIF-1a .

c. Significance. Prostate cancer is the most common primary cancer among men and the second leading cause of cancer deaths in males in the United States. Our previous studies have shown that NEP loss is involved in the development and progression of both early and advanced hormone refractory PC. Moreover, we now show that complete loss of NEP expression in primary PC is associated with a shorter time to PSA recurrence, suggesting that in addition to NEP loss contributing to neuropeptide-mediated PC progression, it permits more effective malignant angiogenesis. Understanding the molecular events involved in the development and progression of PC is critical to developing more effective therapies. These studies, leading to a better understanding of the involvement of NEP in the development and progression of PC, may ultimately provide support for novel antioangiogenic approaches for the treatment of advanced PC as may be relevant in other vascular tumor models such as angiosarcoma,.

d. Plans. Our continuing objective is to elucidate and to understand the involvement of NEP with regard to its anti-angiogenic action. In the third year of grant support, we will continue our studies aimed at understanding the mechanisms of NEP induced inhibition of angiogenesis. Our studies have provided with a better understanding of the role NEP plays in inactivating bFGF. We will continue to focus our functional analysis of NEP in vascular endothelial cells, which when deficient in NEP are capable of forming tumors in soft agar We will pursue studies to better understand the inhibitory role that heparin sulfate proteoglycans play in modulating the bFGF cleaving activity of NEP. In addition, we will use the NEP null murine Lewis Lung Carcinoma Model to better understand the anti-angiogenic role of NEP *in vivo*.

e. Key Research Accomplishment

1. **Goodman, Jr. O.B**., Febbraio M., Simantov R., Zheng R., Shen R., Silverstein R.L., Nanus DM. (2006) Neprilysin inhibits angiogenesis via proteolysis of fibroblast growth factor-2. *J Biol Chem.* 281(44):33597-605

f. Conclusions

- NEP inactivates bFGF by proteolytic cleavage near its C-terminus, a novel antiangiogenic mechanism

- bFGF cleavage products are functionally inactive on vascular endothelial cells due their inability to bind to engage surface receptor and signal through mitogen activated protein kinase.

- NEP deficient murine vascular endothelial cells form colonies in soft agar and have increased basal ERK phosphorylation.

- Hypoxia negatively regulate NEP activity in endothelial and PC cells

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Faham, S., Hileman, R.E., Fromm, J.R., Linhardt, R.J. and Rees, D.C. (1996) Heparin structure and interactions with basic fibroblast growth factor. *Science*, **271**, 1116-1120.

Kajio, T., Kawahara, K. and Kato, K. (1992) Stabilization of basic fibroblast growth factor with dextran sulfate. *FEBS Lett*, **306**, 243-246.

Sommer, A. and Rifkin, D.B. (1989) Interaction of heparin with human basic fibroblast growth factor: protection of the angiogenic protein from proteolytic degradation by a glycosaminoglycan. *J Cell Physiol*, **138**, 215-220.

Appendix

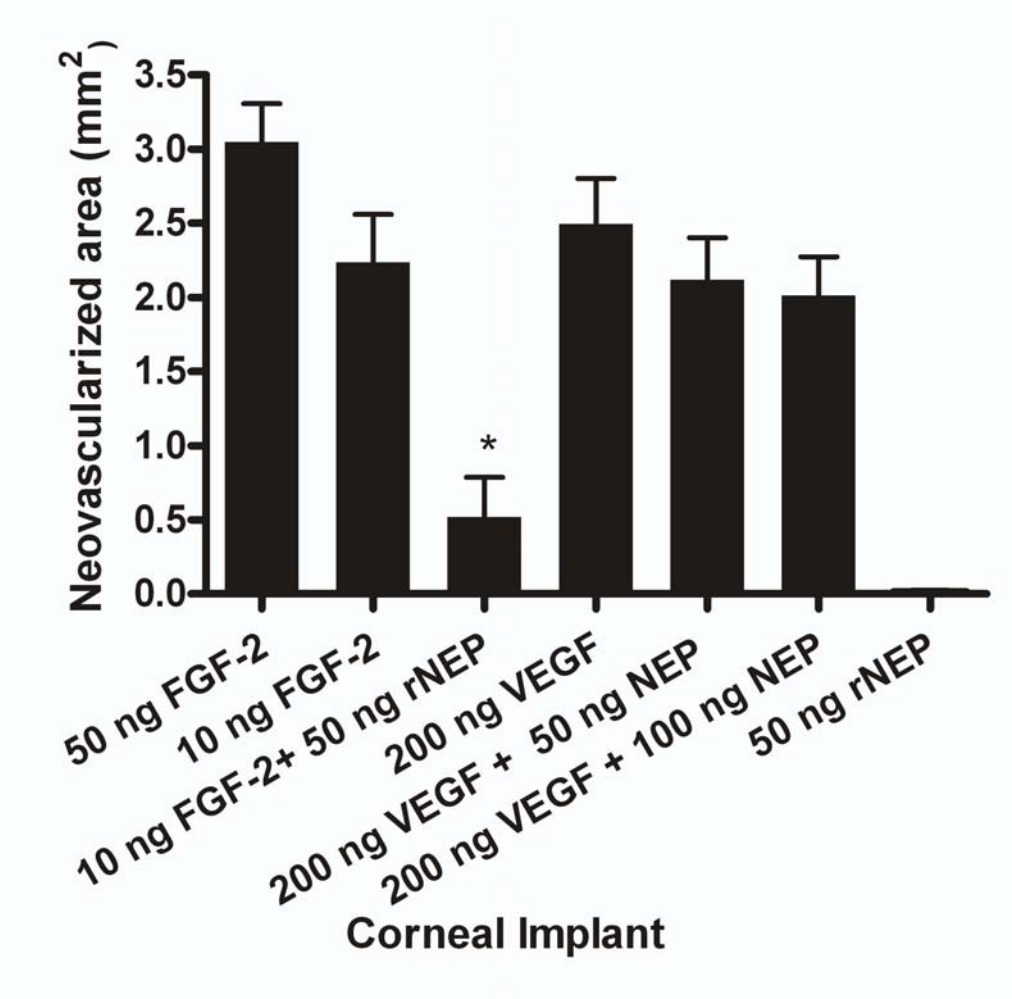

Figure 1**.** Recombinant neprilysin inhibits FGF-2-induced but not VEGF-induced angiogenesis. Hydron pellets containing 50 ng FGF-2 (positive control), 10 ng FGF-2, 10 ng FGF-2 + 50 ng rNEP, 200 ng VEGF, 200 ng + 50 ng rNEP, 200ng + 100 ng rNEP, or 50 ng rNEP alone (negative control) were implanted in the cornea of C57/B6 mice and new vessel formation at 5 days measured by slit lamp ophthalmoscopy. Statistical analysis of six eyes (3 mice) in two independent experiments (a total of 6 mice for each group) was performed (* denotes $P<0.01$ compared to 10 ng FGF-2, 2-tailed unpaired t-test).

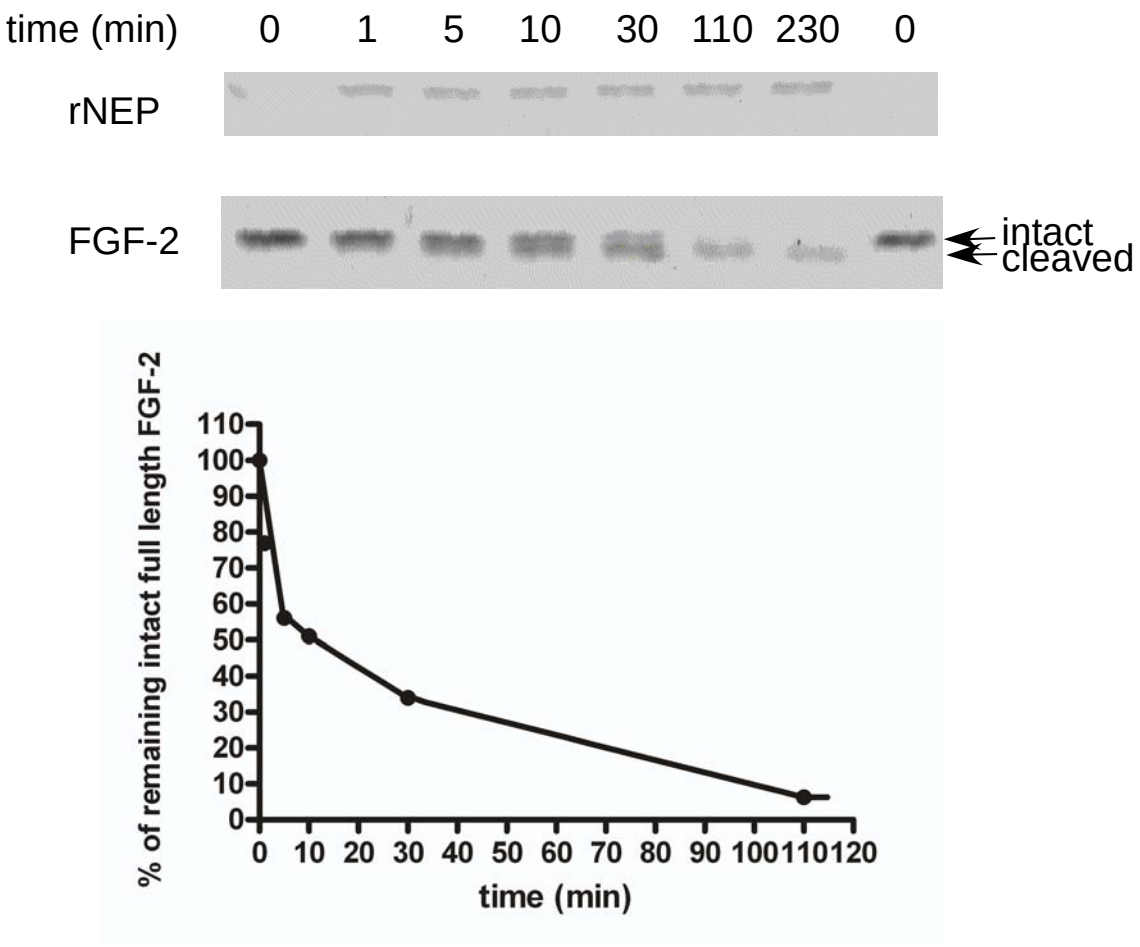

Figure 2. FGF-2 (12 μM) and neprilysin (400 nM) were incubated in 50 mM Hepes pH7.4, 100 mM NaCl and aliquots removed at the various times (1-230 minutes), quenched with SDS sample buffer, and analyzed by SDS-PAGE. Coomassie-stained intact FGF-2 (upper arrow on right) was then quantified densitometrically (NIH ImageJ software) and expressed graphically as the percentage of intact FGF-2, with 100% defined as the intensity of the intact FGF-2 band at time 0, just prior to addition of rNEP.

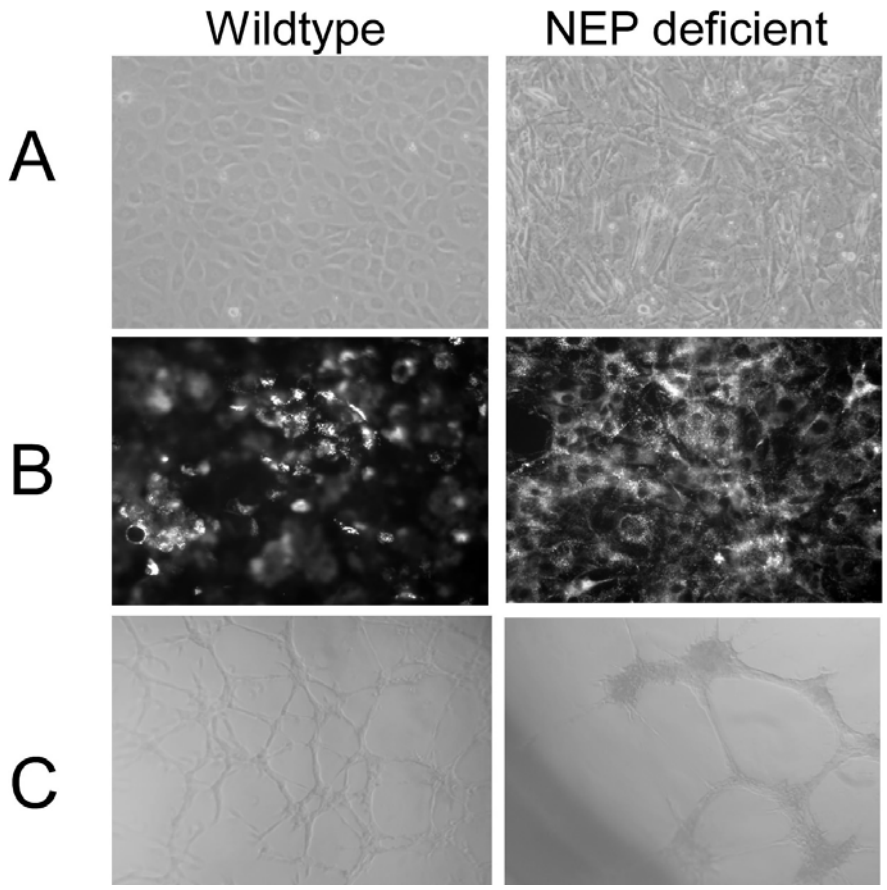

Figure 3. Wildtype (left column) and NEP-deficient (right column) murine cardiac endothelial cell primary cultures. A Primary cell cultures on gelatin grown to confluence. Note the bizarre morphology exhibited by the NEP deficient B. DiI-LDL staining of confluent cardiac endothelial cell cultures. C Cardiac endothelial cells plated on matrigel (50,000 cells on 50 μL of matrigel in a 96 well plate) are capable of forming arrays.

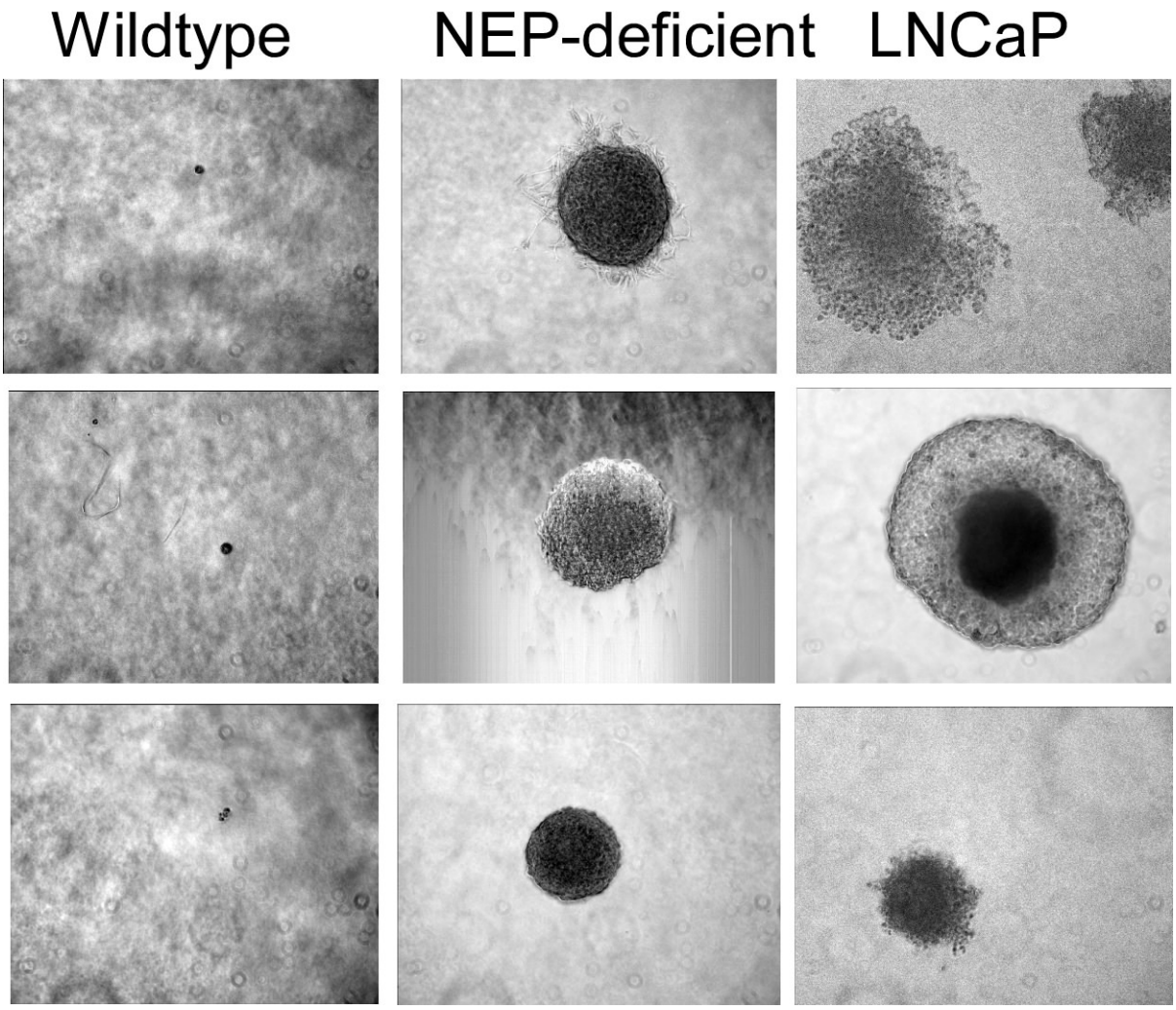

Figure 4. Anchorage-independent growth of NEP deficient murine cardiac endothelial cells. Cells (2500/well) were plated on a layer of 4% agarose containing complete endothelial cell medium or in the case of LNCaP (used as a positive control), RPMI 1640 medium supplemented with 10% FBS, and sandwiched with another. 3 weeks later, wells were photographed. Results are representative of 3 individual experiments each performed in duplicate.

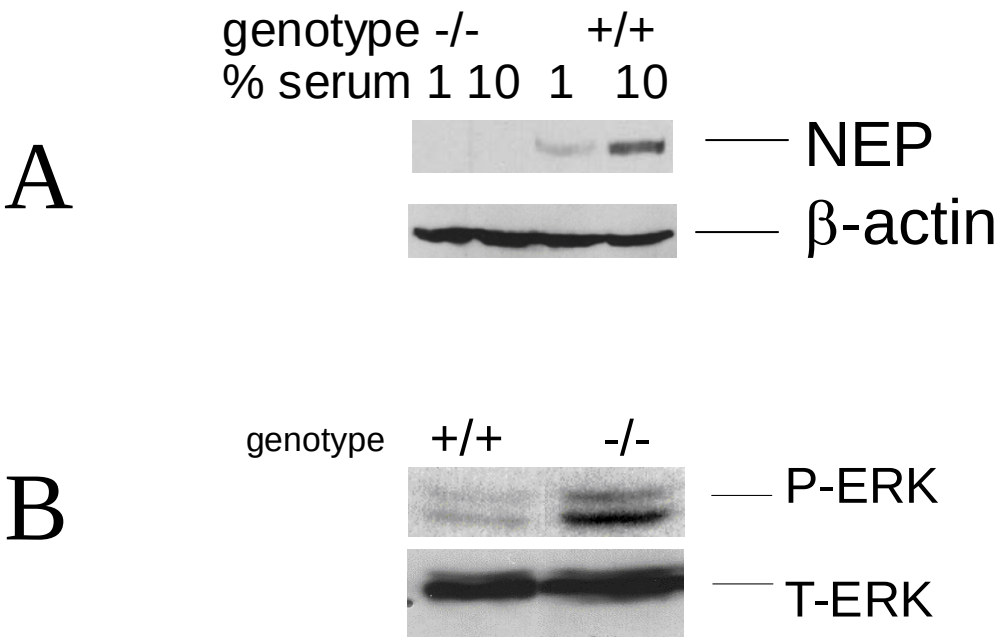

Figure 5. Western blot analysis of CEC cell lysates . A. NEP protein detected by NCl antibody as a function of serum concentration. B. Blotting for phospho-ERK in lysates derived from cells cultured in 2% serum. 50 μg of protein were loaded in each lane.

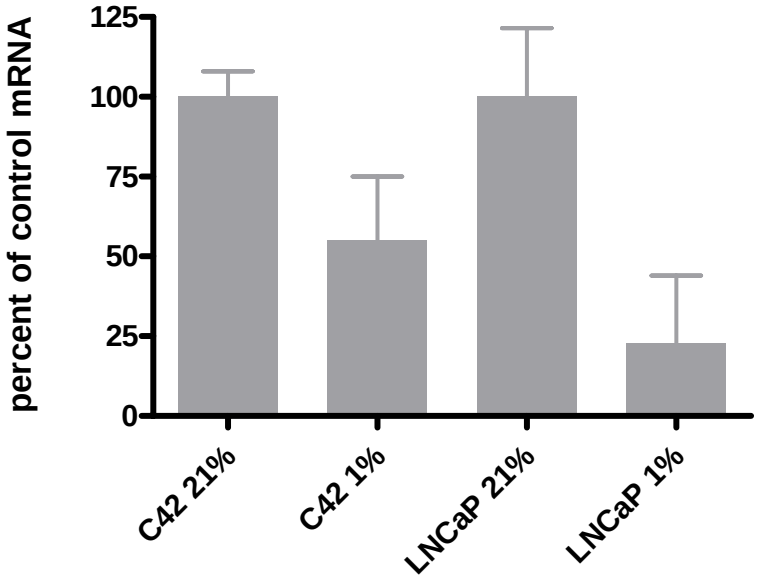

Figure 6. Quantitative real time PCR analysis NEP transcripts as a function of oxygen tension in C42 and LNCaP prostate cancer cell lines cultured under normoxic conditions (21% oxygen tension) or in a sealed hypoxia chamber (1% oxygen tension) for 24 hours. NEP transcripts were analyzed following RT-PCR and normalized using β-actin transcript levels. NEP transcript levels at 21% oxygen tension are arbitrarily set to 100%.